Plasmin Induces Cyr61 Gene Expression in Fibroblasts Via Protease-Activated Receptor-1 and p44/42 Mitogen-Activated Protein Kinase–Dependent Signaling Pathway

Usha R. Pendurthi, Mylinh Nguyen, Patricia Andrade-Gordon, Lars C. Petersen, L. Vijaya Mohan Rao

Objective—The plasminogen system has been proposed to participate in vascular remodeling and angiogenesis. Although plasmin-mediated proteolysis could contribute these processes, proteolytic targets for plasmin and their downstream effector molecules are yet to be fully defined. The aim of the present study was to elucidate potential mechanisms by which plasmin affects various cellular processes.

Methods and Results—Plasmin upregulated the expression of Cyr61, a growth factor–like gene that has been implicated in cell proliferation, adhesion, and migration. Plasmin-induced gene expression is dependent on its proteolytic activity and requires its binding to cells. Studies that used wild-type fibroblasts and fibroblasts derived from PAR-1− and PAR-2−/− deficient mice showed that plasmin induced Cyr61 gene expression in wild-type fibroblasts and PAR-2−/− deficient cells but not in PAR-1−/− deficient cells. Consistent with this, plasmin induced the activation of p44/42 mitogen-activated protein kinase in wild-type, PAR-2+/− cells but not in PAR-1−/− cells. In contrast with thrombin, plasmin failed to induce Ca²⁺ signaling in fibroblasts.

Conclusions—Plasmin induced an angiogenic and wound-healing promoter, Cyr61, in fibroblasts through activation of PAR-1. Plasmin-induced Cyr61 expression is mediated via the p44/42 mitogen-activated protein kinase pathway independent of Ca²⁺ signaling. (Arterioscler Thromb Vasc Biol. 2002;22:1421-1426.)

Key Words: plasmin  ■  Cyr61  ■  protease-activated receptors

Proteases involved in coagulation and fibrinolysis, in addition to their specific role in maintaining hemostasis, play an important role in wound healing, tissue remodeling, and angiogenesis by modulating various cellular processes, such as cell proliferation and migration.1 A number of mechanisms have been proposed as to how these proteases are capable of modulating various cellular processes. Thrombin, the principal effector protease of the coagulation cascade, is thought to elicit cellular responses mainly through the activation of G protein–coupled protease-activated receptors (PARs),2,3 whereas the plasminogen/plasmin system is shown to affect various cellular processes primarily through proteolysis of extracellular matrix (ECM), either directly or indirectly, via the activation of matrix metalloproteinases.4,5 Plasmin-mediated activation of latent growth factors, such as transforming growth factor-β, and the local fibrin clearance can also contribute to the plasmin’s role in tissue remodeling. A review of studies in fibrinolytic system gene-deficient mice indicates that the substrate specificity of plasmin is highly diverse in vivo and dependent on the location and extent of the pathology.6 More importantly, these studies also implicate that plasmin may have significant biological roles, which are yet to be defined, aside from proteolysis of fibrin gel and extracellular matrix.7-9

Our recent studies have shown that clotting proteases factor VIIa and thrombin upregulate the expression of Cyr61 and connective tissue growth factor (CTGF), growth factor–like genes, in fibroblasts.10 Cyr61 and CTGF are immediate-early response genes and belong to a novel class of cysteine-rich–secreted protein family.11 Recent studies have shown that these gene products act as ECM-associated signaling molecules. They are involved in regulation of growth and differentiation in endothelial cells, fibroblasts, and tumor cells and have been found to participate in a wide array of important biological processes, including angiogenesis, wound healing, fibrosis, and atherosclerosis.12-16 Many functions of Cyr61 and CTGF are quite similar to those attributed to plasmin, raising the possibility that they could be linked. Such a link could explain how plasmin could affect various cellular processes independent of its direct action on fibrin and ECM degradation.

In the present study, we investigated whether plasmin could upregulate the expression of Cyr61 in fibroblasts and a possible mode of its action.

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Proteases on Gene Induction

Effect of Various Clotting and Fibrinolytic Proteases on Gene Induction

Our recent studies have shown that thrombin and VIIa/TF, through a protease activity–dependent signaling mechanism, induced the expression of Cyr61 in fibroblasts. To test whether other proteases involved in clotting and fibrinolysis could also induce the expression of Cyr61, quiescent monolayers of human fibroblasts (WI-38 cell line) were treated with various clotting and fibrinolytic proteases (20 nmol/L each) for 45 minutes, and the total RNA harvested from these cells was analyzed for Cyr61 mRNA steady-state levels by Northern blot analysis. The data revealed, in addition to VIIa and thrombin, that FXa, FIXa, and activated protein C (APC) treatments also induced the expression of Cyr61, whereas tissue-type plasminogen activator and urokinase-type plasminogen activator (uPA) had no effect (see online Figure IA). Tissue factor, a specific inhibitor of FXa, and hirudin failed to inhibit the plasmin-induced response (Figure 1A and 1B), suggesting that the thrombin that was present in these proteases as an undetectable trace contaminant or generated on the cells was responsible for the FXa-, FIXa-, and APC-induced response.

Among the fibrinolytic proteases tested, plasmin induced the expression of Cyr61, whereas tissue-type plasminogen activator and urokinase-type plasminogen activator (uPA) had no effect (see online Figure IA). Tissue factor, a specific inhibitor of FXa, and hirudin failed to inhibit the plasmin-induced response (Figure 1A and 1B), suggesting that the plasmin-induced response was generated independently of FXa and thrombin. Furthermore, treatment of cells with recombinant plasminogen (50 nmol/L) activated with recombinant two-chain uPA (0.02 nmol/L) resulted in the induction of Cyr61 expression, whereas treatment of the cells with recombinant plasminogen or uPA alone had no effect (data not shown). Plasmin treatment upregulated the expression of Cyr61 in a time- and dose-dependent manner. The plasmin-induced expression was visible at 30 minutes, peaked at 45 minutes, and returned to the base level at the end of 90 minutes (see online Figure IIA, which can be accessed at http://atvb.ahajournals.org). As little as 2.5 nmol/L plasmin was sufficient to induce the expression of Cyr61, and the maximum response was observed at 25 to 50 nmol/L of plasmin (see online Figure IIB), a concentration equivalent to 1% to 2% of plasma plasminogen.

Plasmin Protease Activity Is Directly Responsible for the Plasmin-Induced Gene Induction

To establish that plasmin protease activity is required for the induction of Cyr61, WI-38 cells were treated with plasmin or PPACK-plasmin (active site–inhibited plasmin) for 45 minutes, and the expression of Cyr61 was evaluated by Northern blot analysis. As shown in Figure 1C, PPACK-plasmin, in contrast to plasmin, failed to induce the expression of Cyr61. Similarly, inhibition of plasmin protease activity by pretreating plasmin with a specific plasma protease inhibitor, α2-antiplasmin, completely abolished the plasmin-induced response (Figure 1C). These data suggest that plasmin protease activity is required for the plasmin-induced gene expression. To examine whether plasmin activation of promatrix metalloproteinases might be responsible for plasmin-induced gene expression, we included matrix metalloproteinase inhibitors, GM 1489 and GM 6001, in the incubation medium. As shown in Figure 1D, these inhibitors failed to block the plasmin-induced response. Furthermore, treatment of the cells with elastase and collagenase had no or only a minimal effect on the expression of Cyr61 (data not shown). These data suggest that the protease activity of plasmin is directly responsible for the induction of Cyr61 gene expression in fibroblasts. Tranexamic acid, which inhibited plasmin binding to the cells (data not shown), completely inhibited the plasmin-induced response (Figure 1E).

Plasmin-Induced Gene Expression Involves the Activation of PAR-1

Because the protease activity of plasmin is required for the gene induction and our earlier studies show that the activation of PAR-1, and to a lower extent PAR-2, could result in the induction of Cyr61 expression in fibroblasts, we next investigated whether plasmin-mediated gene induction in-
involves the activation of PAR-1 or PAR-2. First, WI-38 cells were treated with specific neutralizing antibodies against PAR-1 before they were exposed to plasmin. Pretreatment of cells with PAR-1-specific antibodies completely abolished the plasmin-induced gene expression (Figure 1F), suggesting plasmin-induced gene expression is mediated via PAR-1. In a second set of experiments, fibroblasts derived from wild-type mouse, PAR-1, or PAR-2 knockout mice were treated with plasmin or other agonists, and the induction of CyR61 expression was analyzed by Northern blot analysis using mouse CyR61 cDNA probe. Plasmin induced the expression of CyR61 in wild-type mouse fibroblasts (Figure 2A) and PAR-2 −/− fibroblasts (Figure 2C) but not in PAR-1 −/− cells (Figure 2B). Next, we investigated the effect of plasmin in PAR-1 −/− cells transfected with human PAR-1 or PAR-2 cDNA to express functional PAR-1 or PAR-2. As shown in Figure 3A, plasmin effectively induced the gene expression in PAR-1 −/− cells that were transfected with PAR-1. Plasmin also could induce CyR61 expression in PAR-1 −/− cells expressing PAR-2; however, the effect was moderate, approximately a 2- to 3-fold increase over the control (untreated) cells (Figure 3B). In these cells, trypsin, a known PAR-2 activator, effectively induced CyR61 expression.

In additional experiments, we treated PAR-2 −/− cells with both plasmin and thrombin for 45 minutes and CyR61 expression was evaluated. Data from these experiments revealed that plasmin and thrombin effects are not additive (relative levels of CyR61 mRNA = control, 100%; plasmin, 1013%; thrombin, 1217%; plasmin + thrombin, 1076% [mean of two experiments]). Further experiments showed that plasmin-induced CyR61 gene expression in PAR-2 −/− cells is correlated with the increased CyR61 antigen levels (see online Figure III, which can be accessed at http://atvb.ahajournals.org). Overall, these data confirm that the plasmin induces gene expression in fibroblast primarily through a PAR-1-dependent pathway.

Plasmin Does Not Induce Cytosolic Ca2+ Release in Fibroblasts

Because activation of PARs was shown to invariably result in intracellular calcium mobilization and to determine the role of the Ca2+-signaling pathway in plasmin-induced gene induction, we investigated the effect of plasmin on cytosolic Ca2+ release. A concentration of plasmin (20 nmol/L) that effectively induced CyR61 gene expression failed to produce a detectable Ca2+ flux in WI-38 cells (Figure 4A). Increasing plasmin concentration to 100 nmol/L also had no effect on the calcium mobilization (Figure 4B). As expected, thrombin treatment resulted in a prominent increase in intracellular Ca2+ concentration in these cells (Figure 4A). In addition to thrombin, PAR-1− and PAR-2−specific peptide agonists also induced Ca2+ release (Figure 4B). Overall these data suggest that PAR-1 (and PAR-2)−mediated Ca2+-signaling pathway is intact in these cells; however, plasmin does not activate this pathway in fibroblasts.

**Figure 2.** Plasmin-induced CyR61 expression requires the presence of PAR-1. Quiescent monolayers of wild-type (A), PAR-1 −/− (B), and PAR-2 −/− (C) mouse fibroblasts were treated with a control medium alone or the medium supplemented with plasmin (50 nmol/L), PAR-1 peptide agonist (PAR-1-P; 50 µmol/L), PAR-2 peptide agonist (PAR-2-P; 50 µmol/L), thrombin (8.5 nmol/L [1 U/mL]), or serum (10% v/v) for 45 minutes. At the end of treatment, total RNA was harvested, and 10 µg of total RNA was subjected to Northern blot analysis. The Northern blots were probed with radiolabeled mouse CyR61 cDNA probe. The upper panels show representative autoradiographs and the bottom panels show quantitative data (mean±SE, n=3 to 7). Asterisk mark in bar graph (A) denotes the data were mean values from two experiments. CyR61 expression in serum-treated cells was 25- to 100-fold higher in comparison with untreated cells, which was not included in the bar graph that shows quantitative data to illustrate the lower magnitude effects of plasmin and other agonists.

**Figure 3.** Effect of plasmin in PAR-1 −/− cells stably transfected to express PAR-1 or PAR-2. Quiescent monolayers of PAR-1 −/− cells expressing human PAR-1 or PAR-2 were treated with a control medium or the medium supplemented with one of the following agonists: plasmin (50 nmol/L), PAR-1 peptide agonist (PAR-1-P; 50 µmol/L), PAR-2 peptide agonist (PAR-2-P; 50 µmol/L), thrombin (8.5 nmol/L), trypsin (50 nmol/L), or serum (10% v/v) for 45 minutes. At the end of treatment, total RNA was harvested, and 10 µg of total RNA was subjected to Northern blot analysis. The blot was probed with radiolabeled mouse CyR61 cDNA probe. The upper panels show representative autoradiographs and the bottom panels show quantitative data (mean±SE, n=3 to 5). Quantification of CyR61 expression in serum-treated cells was not included in the bar graph.
Plasmin Induces p44/42 Mitogen-Activated Protein Kinase (MAPK) Activation in Fibroblasts

To determine whether plasmin-induced gene expression involves the activation of p44/42 MAPK, we first determined the effect of plasmin on p44/42 activation in WI-38 cells as well as mouse PAR-2 \(-/-\) cells. The cells were treated with plasmin (50 nmol/L) for varying time periods, and the phosphorylation of p44/42 MAPK was analyzed by Western blot analysis. Similar results were obtained with both cell types, and the data obtained with PAR-2 \(-/-\) cells are shown in Figure 5. The data show that plasmin induced p44/42 MAPK activation in a time-dependent manner. Increased phosphorylation of p44/42 MAPK was evident as early as 2 minutes after exposure to plasmin, reaching maximum levels at 10 to 20 minutes and declining thereafter (Figure 5). Treatment of WI-38 cells for 20 minutes with varying concentrations of plasmin revealed that a 25 nmol/L concentra-
tion of plasmin, a concentration that maximally induced Cyr61 expression, also maximally induced p44/42 activation (data not shown). In contrast with WI-38 cells and PAR-2 \(-/-\) cells, plasmin failed to induce p44/42 MAPK activation in PAR-1 \(-/-\) cells (Figure 5).

Effect of Various Intracellular Signaling Pathway Inhibitors on Plasmin-Induced Gene Expression

To test the hypothesis that plasmin-induced gene expression is mediated via p44/42 MAPK activation and to obtain clues on upstream signaling events involved in the plasmin-induced gene expression, we investigated the effect of various intracellular signaling inhibitors on plasmin-induced Cyr61 expression. PD98059 and U0126, selective inhibitors for MAP kinases (MEKs), reduced the plasmin-induced gene expression by 65% and 40%, respectively (see online Figure IV, which can be accessed at http://atvb.ahajournals.org). Genistein, a protein tyrosine kinase inhibitor, markedly reduced the plasmin-induced gene expression. In addition, phospholipase C inhibitor, U73122, suppressed the plasmin-induced gene expression by more than 50%. Specific inhibitors for phosphatidylinositol-3 (PI-3) kinase, wortmannin, and LY 294402 inhibited the plasmin-induced gene expression by about 75% and 100%, respectively. Cholera toxin, a modulator of G protein signaling, also completely blocked the plasmin-induced gene expression.

Discussion

The data presented herein demonstrate that plasmin, a pro-
tease best known for its ability to cleave fibrin and matrix metalloproteinases, induces p44/42 MAPK activation and upregulates Cyr61 gene expression in fibroblasts. We believe that this is the first report that shows plasmin induces expression of a growth factor–like gene in fibroblasts, which could potentially contribute to fibroblast proliferation and migration. Our data also establish that plasmin-induced responses are primarily mediated through PAR-1. Although the ability of plasmin to activate cells by PAR-1 cleavage has been shown in earlier studies,\(^{17-19}\) an important difference between the present study and earlier studies is the concentration of plasmin required to activate PAR-1 to induce gene expression and affect cellular processes. Earlier studies showed very high concentrations of plasmin (300 to 800 nmol/L) were needed to cleave PAR-1\(^{20}\) and to activate platelets moderately.\(^{17,18}\) In contrast, the data presented herein show that low concentrations of plasmin (as low as 2.5 nmol/L) effectively induce the gene expression in fibroblasts.

The evidence that plasmin-induced signaling is mediated via PAR-1 comes from the following observations: (1) plasmin induces the expression of Cyr61 in human fibroblasts that express PAR-1, (2) PAR-1–specific antibodies block the plasmin-induced gene expression, (3) mouse wild-type myo-

fibroblasts that express PAR-1 but not other PARs respond to plasmin, (4) plasmin fails to induce the gene expression in myofibroblasts derived from PAR-1 knockout mice (PAR-1 \(-/-\) cells) although it effectively induces the gene expression in myofibroblasts derived from PAR-2 knockout mice (PAR-2 \(-/-\) cells), (5) PAR-1 \(-/-\) cells transfected with PAR-1 expression vector respond to plasmin to induce gene expression.
expression, and (6) plasmin induces the activation of p44/42 MAPK in wild-type and PAR-2−/− cells but not in PAR-1−/− cells.

The in vitro kinetic studies, which used various lengths of exodomain of PAR-1, reported that plasmin cleaves at R41 site with 10- to 1700-fold lower efficiency compared with thrombin. Furthermore, plasmin was shown to cleave PAR-1 rapidly at multiple sites downstream of the activation site, which would disable PAR-1 by the removal of the tethered ligand. Such a cleavage pattern explains the seemingly contradictory earlier data on the effect of plasmin on platelet activation and 

Camerer et al showed that the threshold concentration of FXa for detecting FXa-induced PAR-1−mediated signaling was 7 nmol/L, and the FXa response was not saturated even at 870 nmol/L Xa, suggesting FXa is a weak activator of PAR-1. Furthermore, the plasma concentration of plasminogen (the zymogen form for plasmin; 2.4 μmol/L) was substantially higher than that of factor X (0.18 μmol/L). Thus, it is more likely that, compared with FXa, plasmin could be a more potent activator of PAR-1 in vivo.

The failure to detect intracellular Ca2+ mobilization with low concentrations of plasmin used in the present study could be due to an inefficient cleavage of PAR-1 by plasmin. Earlier studies showed that the magnitude of protease response mediated by PAR-1 is determined by both the rate and extent of receptor cleavage. At a lower concentration of protease or if the protease is not very efficient, only a limited number of receptors would be activated. Although the limited number of activated receptors could transduce signals, a measurable response requires activation of a minimum number of receptors at certain rate. The rate and extent of receptor activation required to produce the response may depend on the response that would be measured. For example, when receptor cleavage correlated with phosphoinositide hydrolysis, inositol trisphosphate (IP3) formation was proportional to the absolute amount of cleaved receptor, but the subsequent increase in cytosolic Ca2+ occurred only if IP3 was generated quickly enough to accumulate. Such a scenario may explain why we observed dose-dependent plasmin-induced gene expression with 2.5 to 50 nmol/L of plasmin, whereas as high as 100 nmol/L of plasmin failed to show an increase in Ca2+ uptake. In this context, it may be pertinent to point out that FXa, which is also a poor activator of PAR-1, failed to produce Ca2+ flux under the similar experimental conditions (data not shown). Alternatively, the present data could also be interpreted that plasmin-induced activation of PAR-1 does not result in an increased cytosolic Ca2+ levels.

The efficient cleavage of PAR-1 by thrombin comes from the mode of recognition of PAR-1 by thrombin. PAR-1 has hirudin-like sequences. This allows thrombin to recognize PAR-1 as a substrate. Unlike thrombin, the major determinant for FXa cleavage of PAR-1 seems to be the cell surface localization of FXa in proximity to the receptor rather than sequence-specific recognition of the cleavage site. At present, it is unknown what the major determinants for plasmin cleavage of PAR-1 are. Because plasmin cleaved the entire exodomain of PAR-1 with much higher efficiency than it cleaved the exodomain peptide containing L38-E60, it has been suggested that plasmin recognizes binding determinants located to the N-terminal side of L38. In addition to structural and binding determinants in PAR-1, it is possible that additional cell-surface components may also contribute to plasmin activation of PAR-1. The observation that tranexamic acid, a lysine analogue, which blocks plasmin binding to cells, completely inhibits the plasmin-induced response fits with this hypothesis. Further support for this hypothesis comes from the observation that plasmin fails to induce Cyr61 gene expression in COS-7 cells, which contain functional PAR-1 (see online Figure V, which can be accessed at http://atvb.ahajournals.org).

At present, the signaling pathways that are involved in plasmin-induced gene expression are yet to be fully defined. However, our present data provide important clues on this subject. Plasmin-induced Cyr61 gene expression is fully suppressed by cholera toxin, supporting the involvement of a G protein, Gs, in plasmin-induced gene expression. U73122, a PI-PLC inhibitor, and LY294002, a specific PI-3 kinase inhibitor, markedly diminished the plasmin-induced response, suggesting a role for the second messenger IP3 in mediating the plasmin-induced expression. Because plasmin, even at a 10-fold higher concentration than that was required to induce gene expression, failed to increase cytosolic Ca2+ levels in fibroblasts, it is unlikely that the plasmin-induced gene expression involves a Ca2+-signaling pathway. The observations that plasmin activates p44/42 MAPK and the specific inhibitors of p44/42 MAPK abrogate the plasmin-induced gene expression suggest that the plasmin-induced gene expression is mediated via the p44/42 MAPK pathway. Overall, these data suggest that the plasmin-induced gene expression is mediated via PI-PLC through MEK and ERK.

Irrespective of the mechanism by which plasmin induces the gene expression, the observation that plasmin induces the expression of Cyr61 adds another dimension to our understanding of how plasmin regulates various cellular processes,
particularly angiogenesis and tissue remodeling. Cyr61, an extracellular signaling protein, is shown to influence a wide array of important biological processes, including promotion of cell proliferation, adhesion, and migration of endothelial cells and fibroblasts.\textsuperscript{11} Expression of Cyr61 in tumor cells was shown to promote tumor growth and vascularization.\textsuperscript{31} A recent study showed that Cyr61 in skin fibroblasts regulates a number of genes that encode proteins that control angiogenesis, inflammation, ECM remodeling, and cell-ECM interactions.\textsuperscript{32} It is interesting to note that Cyr61-induced genes include matrix metalloproteinase (MMP)1, MMP3, uPA, and PAI-1, whose activities are either regulated by plasmin or regulate plasmin generation. At present, plasmin is thought to play a role in tissue remodeling by degrading ECM, either directly or indirectly through the activation of certain pro-MMPs.\textsuperscript{4,5} The ability of plasmin to upregulate the expression of Cyr61, which in turn could upregulate the expression of MMPs and other genes involved in angiogenesis and matrix remodeling suggest that plasmin plays a crucial role in angiogenesis and tissue remodeling by multiple mechanisms in a tightly regulated manner. The ability of plasmin to influence these processes via Cyr61 would allow plasmin to sustain its effects well beyond after its proteolytic activity is inhibited by plasma inhibitors. The ability of plasmin to induce Cyr61 and probably other growth factor–like molecules, in addition to degrading ECM and activating growth factors, would provide a coordinated spatiotemporal effect that is required for angiogenesis, wound healing, and tissue remodeling. Further studies, using appropriate model systems, are needed to test the importance of plasmin-induced gene expression in pathophysiology.

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Plasmin induces *Cyr61* gene expression in fibroblasts via protease activated receptor-1 and p44/42 MAPK-dependent signaling pathway

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Methods

Cells
A human fibroblast cell line (WI-38) derived from normal embryonic lung tissue was obtained from ATCC, Rockville, MD. Immortalized PAR-1 deficient (PAR-1-/-) and PAR-2 deficient (PAR-2 -/-) murine lung myofibroblasts were derived from PAR-1\textsuperscript{1} and PAR-2\textsuperscript{2} knock-out mice. Immortalized wild-type murine lung myofibroblasts were derived from the same strain of mouse that was used to generate the knock-out mice. PAR-1-/- cells, which lack all functional PARs, were transfected with human PAR-1 or PAR-2 cDNA using a mammalian expression vector encoding hygromycin resistance gene (pcDNA3.1). All cell lines were grown in Dulbecco's modified Eagle medium (GIBCO BRL Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Cellgro, Herndon, VA), 1% penicillin and streptomycin (Bio Whittaker, Walkersville, MD), and 1% L-glutamine (Bio Whittaker). The medium was supplemented with hygromycin B (250 µg/ml) when transfected cell lines were cultured.

Proteins and Other Reagents
Coagulant and fibrinolytic proteases, and the corresponding active site-inhibited proteases were obtained from either Haematological Technologies, Inc. (Essex Junction, VT) or Enzyme Research Laboratories (South Bend, IN). The proteases were apparently homogenous, as judged by SDS-PAGE. Amidolytic activity of plasmin preparations was not reduced by hirudin, but completely inhibited by α2-antiplasmin, suggesting that plasmin preparations were devoid of any measurable amounts of thrombin or other proteases. Active site-inhibited plasmin was prepared by incubating plasmin with a 100-fold molar excess of D-Phe-L-Phe-L-Arg chloromethyl ketone (Calbiochem, San Diego, CA) for 1 h at 37°C and then removing the free inhibitor by an extensive dialysis at 4°C against 10 mM Hepes, 0.15 M NaCl, pH 7.5. α2-antiplasmin was obtained from American Diagnostica Inc. (Greenwich, CT). Recombinant plasminogen and recombinant two-chain uPA were kindly provided by Dr. Doug Cines laboratory (University of Pennsylvania, PA). Recombinant tick anticoagulant protein (TAP) was provided by Dr. George Vlasuk...
PAR-1 peptide agonists, SFLLRN and TFLLRN, and PAR-2 peptide agonist, SLIGRL, were custom synthesized as carboxy-amide peptides (Biosynthesis Inc., Lewisville, TX). Hirudin, protein kinase inhibitors and other inhibitors were purchased from Calbiochem. Antibody kit for analysis of phosphorylation of p44/42 MAP kinase was obtained from Cell Signaling Technologies (Beverly, MA). Rabbit anti-mouse Cyr61 antiserum was obtained from Munin Corporation (Chicago, IL). PAR1 antibodies WEDE15 were obtained from Beckman Coulter (Fullerton, CA) and ATAP2 were from Santa Cruz (Santa Cruz, CA).

**Northern Blot Analysis**

Total RNA was prepared from quiescent monolayers of WI-38 or mouse fibroblasts that were exposed to plasmin or other compounds as described in RESULTS. TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) was used to isolate total RNA and the RNA was dissolved in RNAsecure™ (Ambion, Austin, TX). Northern blot analysis was performed with $^{32}$P-labeled human or mouse Cyr61 cDNA probes essentially as described earlier. The hybridized membranes were exposed to Kodak X-Omat Blue film and the autoradiographs were scanned. In few figures, lanes were rearranged within the autoradiograph for systematic presentation of the data. For quantification purposes, the membranes were exposed to phosphor screens for 1 to 4 h, and the exposed screens were analyzed in a PhosphorImager (Molecular Dynamics) using "Image-Quant, NT" software. The phosphor image units (counts) obtained in various treatments were normalized to the units present in the control (non-treated) sample of the same experimental group.

**Western Blot Analysis**

Cells were grown to about 80-90% confluency in 35 mm culture dish (six-well plate) and deprived of serum for 24 hours before they were treated. After specific treatments as described in RESULTS, cells were lysed in 150 µl SDS sample buffer (9.2 % w/v SDS, 25 mM Tris.HCl, 20 % v/v glycerol, 80 mM EDTA, 1.2 % w/v bromophenol blue, pH 6.8) supplemented with 3 mM Na-orthovanadate and 24.2 mM DTT. Cell lysates (15 µl) were loaded on a 12% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane by electroblotting. Phosphorylated and total p44/42 MAP kinases were detected
using a PhosphoPlus™ p44/42 MAPK Antibody kit (Cell Signaling, Beverly, MA) using the protocol recommended by the manufacturer. For a positive control, the cells were treated with 15% FCS for a 10-min period. Cyr61 antigen was detected by the Western blot analysis using a rabbit anti-mouse Cyr61 antibody.

**Measurements of Cytosolic Ca^{2+} release**

WI-38 cells cultured in Lab-Tek chambered cover glass (NUNC Inc., Naperville, IL, USA) were loaded with 0.5 µM Fluo-4/AM (Molecular Probes Inc., Eugene, OR, USA) in a Hepes buffer containing calcium (10 mM HEPES, 140 mM NaCl, 5 mM KCl, 5.5 mM D-glucose, 1 mM MgSO_{4} and 1.5 mM CaCl_{2}) for 30 min at 37°C. The Fluo-4 loaded cells were placed on the temperature-regulated stage of a Diaphot300 microscope (Nikon Corp., Tokyo, Japan) equipped with a PTI photomultiplier tube. The cells were illuminated with a Polychrome I monochromator (T.I.L.L. Photonics GmbH., Planegg, Germany). The light path had a 490 dichroic mirror and a 515±15 nm emission filter. Fluorescence emission at 515 nm of Fluo-4 probe was measured with excitation at 488 nm. The calcium concentration is presented as normalized fluorescence intensity where fluorescence intensity obtained with a buffer control was taken as 1.
References


Figure legends

**Figure I.** Effect of various clotting and fibrinolytic proteases on *Cyr61* gene expression. **Panel A:** Quiescent monolayers of fibroblasts (WI-38 cells) were incubated with various clotting and fibrinolytic proteases (20 nM each) for 45 min. Total RNA was harvested and subjected (10 µg of RNA) to Northern blot analysis with radiolabeled *Cyr61* cDNA. **Panel B:** Factor Xa (2 nM), Factor IXa (20 nM) or APC (20 nM) was incubated with hirudin (5 units/ml) or TAP (200nM) for 30 min before they were added to quiescent monolayers of WI-38 cells. After 45-min treatment period, total RNA was harvested, 10 µg of total RNA was subjected to Northern blot analysis and were probed with radiolabeled *Cyr61* cDNA.

**Figure II.** Time and dose-dependent plasmin-induced expression of *Cyr61*. Quiescent monolayers of WI-38 cells were treated with plasmin (50 nM) for varying time periods (panel A) or with varying concentrations of plasmin for 45 min (panel B). At the end of treatment period, total RNA was harvested and 10 µg of total RNA was subjected to Northern blot analysis and the blots were probed with radiolabeled *Cyr61* cDNA.

**Figure III.** Plasmin-induced *Cyr61* antigen expression in PAR-2 -/- cells. Quiescent monolayers of PAR-2 -/- cells were treated with plasmin (50 nM) for varying time periods. At the end of specific intervals, the supernatant medium was removed, and the cells were extracted in SDS-PAGE sample buffer. An equal amount of cell extracts were subjected to SDS-PAGE and blotted to PVDF membrane. The blot was probed with anti-mouse *Cyr61* antiserum.

**Figure IV.** Effect of various intracellular signaling inhibitors on plasmin-induced expression of *Cyr61*. Quiescent monolayers of WI-38 cells were preincubated with inhibitors for 1 h prior to the addition of plasmin (50 nM). After 45 min-treatment with plasmin, total RNA was harvested, subjected to Northern blot analysis using radiolabeled *Cyr61* cDNA. The concentration inhibitors used were as follows:
PD98059, 50 µM; U0126, 50 µM; U73122, 50 µM; LY294002, 50 µM; genistein, 25 µM; wortmannin, 50 nM and cholera toxin, 1 µg/ml. The top panel shows autoradiographs from a representative experiment, and the bottom panel shows quantitative data (n=4, mean ±S.E.).

**Figure V.** Plasmin fails to induce *Cyr61* expression in COS-7 cells that respond to thrombin and PAR-1 peptide agonist. Quiescent monolayers of fibroblasts were treated for 45 min with a control medium alone or the medium containing plasmin (50 nM), thrombin (1 U/ml) or PAR-1 peptide agonist (TFFLRN) (50 µM). At the end of 45 min, total RNA was harvested, 10 µg of total RNA was subjected to Northern blot analysis and probed with radiolabeled *Cyr61* cDNA probe.
Fig. IA

Fig. IB
Fig. II

Time, min

0  15'  30'  45'  60'  90'  120'

Plasmin, nM

0  1.0  2.5  10  25  50

A

B
Figure III

Plasmin

Control

15 min 1 h 2 h 4 h 6 h

46.5 kDa

37.5 kDa
Figure IV

Plasmin

Control
None
PD98059
U0126
U73122
LY294002
Genistein
Wortmannin
Cholera Toxin

Plasmin

Cyr61 mRNA steady state levels (% control)
Figure V